An Improved HPLC Method for the Determination of Ethionamide in Serum

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Summary: A simple and sensitive HPLC method was developed for the determination of ethionamide (ETA) in serum. The method was based on liquid liquid extraction with acetonitrile causing precipitation of serum proteins, drying of supernatant layer and reconstitution with mobile phase. The separation was done by reverse phase chromatography using a hypersil ODS column (250 mm x 4.6 mm, 5μm particle size) and detection at 291 nm. The mobile phase consisted of acetonitrile: phosphate buffer (75:25) which was delivered at 1.5 mL/ min flow rate. The retention time of ETA was found to be 3.8 min. The assay was linear in the concentration range of 0.1-4 μg/ mL. Within day and between day precision extract by relative standard deviation was less than 4 % and inaccuracy did not exceed 3 %. This method is an improved method wherein instead of solid phase extraction, liquid liquid extraction is carried out which resulted in increased sensitivity.

Introduction

Ethionamide (2-ethyl thioisonicotinamide, ETA) is a nicotinamide derivative and structural analogue of isoniazid (Fig. 1). It is used for the treatment of resistant tuberculosis in which *Mycobacterium tuberculosum* becomes resistant to first line drug [1]. ETA is yellow crystalline powder having melting point in the range of 158-164 °C. It is completely soluble in alcohol, acetonitrile and is sparingly soluble in water [2]. *In vivo,* ETA decreases the number of culturable *Mycobacteria* from the lungs. The multiplication of human strains of *Mycobacteria* is suppressed by ETA at 0.6-2.5 mg/ L. ETA *in vitro* inhibits its multiplication at 0.6-1.2 mg/ L. Bovine strains and BCG are more resistant and are inhibited only at a concentration of 5.0 mg/ L [3, 4]. Few methods have been available for the quantification of ETA in serum/ plasma using HPLC with UV detection as well as LC-MS [5, 6]. Another method is ion interaction reagent (IIR) RP-HPLC for the determination of anti tuberculosis drugs and their metabolites singly or in multi component mixtures in biological fluids [7]. A microbiological method has also been reported using different strains of bacteria [8]. A solid phase extraction (SPE) method has also been published for ETA quantification using octadecyl-SPE column [9]. The present work describes an improved method of this published work [9] wherein we have used liquid liquid extraction which resulted in high extraction yields and hence increased sensitivity of the sample. Therefore, the given method is simple and sensitive with U.V. detection for the determination of ETA in serum.

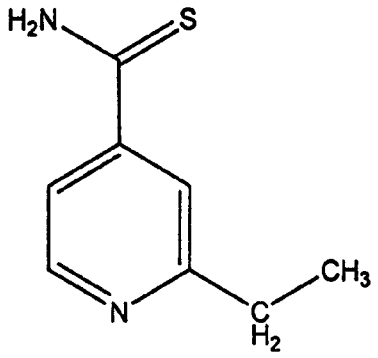

Fig. 1: Structural formula of ethionamide.

Results and Discussion

The calibration curve of ETA constituted in serum demonstrates the concentration vs response (peak area) which was linear in the range of 0.1-4.0 μg/ mL (Fig. 2). The r^2 value was 0.9957. The slope and intercept were calculated as given in Table-1. The intra-day and inter day accuracy and precision for 100 ng/ mL was 97.6 9 % and 98.41 % respectively. For 4000 ng/ mL, these values were 99.39 % and 99.50 % respectively. The intra day and inter-day variability (CV) for 4000 ng/ mL was

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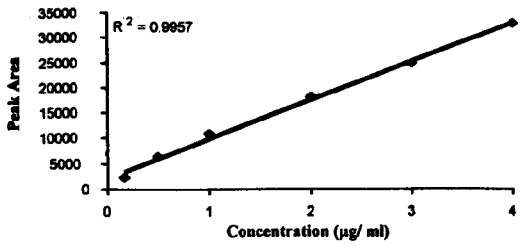

Fig. 2: Standard curve for ETA administered in the oral dose of 250 mg.

Table-1: Mean ± standard deviation, slope, intercept and correlation coefficient of calibration curve equations (n = 5).

Parameters	Mean	S.D.
Slope	0.0043	0.00035
Intercept	0.0057	0.00043
Correlation coefficient	0.9995	0.00062

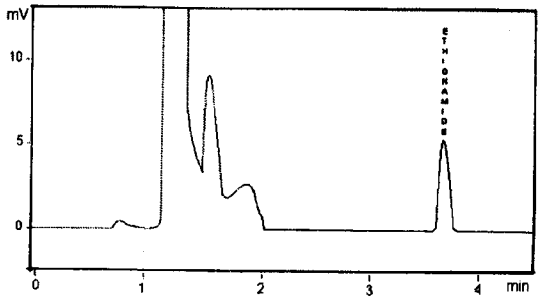

Fig. 3: Chromatogram showing peak of ETA separated on C_{18} column.

0.268 and 0.310, respectively (Table-2 and 3). A typical chromatogram of serum ETA samples is shown in Fig. 3.

In this improved liquid-liquid extraction method, percent extraction yield was calculated by dividing extracted serum samples with serum free dilutions. The average percentage recovery for all the samples was 86.23 ± 2.66 % (Table-4).

In the present method, ETA was extracted from serum using liquid liquid extraction method based on precipitation of serum proteins with acetonitrile. Several methods have been used to accomplish extraction of ETA and its metabolites from biological fluids [5, 7, 9]. Previously, methanol in combination with acetonitrile was used as the extraction solvents using SPE columns for the purpose of extraction of ETA from the serum samples and C_{18} column as analytical stationary phase [9]. Our data shows a clear and non-noisy chromatogram obtained with only acetonitrile as extraction solvent; the percentage recovery from the serum samples was increased up to 86.23 % (Table-

Table-2: Accuracy and precision in serum samples in intra-day performance in duplicates.

Code	A	B	C	D	E
Nominal concentrations of ETA (ng/ mL)	100.0	500.0	1000.0	2000.0	4000.0
Extracted concentrations of ETA (ng/ mL)	102.458	485.675	1024.057	1968.317	3988.524
	95.325	425.698	968.324	1988.214	3968.468
	98.125	472.921	985.317	1996.468	3979.483
	98.653	491.810	972.849	1989.718	3985.986
	95.365	498.240	1014.809	1982.993	3962.856
	96.235	474.856	1005.618	2013.540	3967.427
Mean	97.693	474.866	995.162	1989.875	3975.457
S.D.	2.719	25.967	23.001	14.955	10.676
CV	2.783	5.468	2.311	0.751	0.268
%Accuracy	97.69	94.97	99.52	99.49	99.39

Table-3: Accuracy and precision in serum samples in inter-day performance in duplicates.

Curve Code	A	B	C	D	E
Nominal concentrations of ETA (ng/mL)	100.0	500.0	1000.0	2000.0	4000.0
Extracted concentrations of ETA (ng/mL)	103.882	479.628	992.658	1982.361	3965.208
	94.581	486.213	998.176	1996.108	3988.225
	96.504	485.658	989.604	1986.105	3979.588
	100.259	495.708	1007.253	1994.283	3985.566
	96.584	482.197	992.908	1987.192	3980.736
	98.657	481.808	1004.638	2009.620	3965.693
Mean	98.411	485.202	997.539	1992.611	3980.716
S.D.	3.318	5.713	7.119	9.809	12.377
CV	3.371	1.177	0.713	0.492	0.310
%Accuracy	98.41	97.04	99.75	99.63	99.52

Table-4: Percentage recovery of ETA calculated in three standard dilutions in triplicates.

concentration (ng/ mL)	percentage recovery	S.D.
100	86.31	3.2
500	87.70	2.8
1000	84.69	1.98
Mean	86.23	2.66

4) while the percentage recovery from the SPE method is about 64 %. The method extraction efficiency of this method was found to be higher than previous published methods. The value of r^2 of the calibration curve showed the linearity of the method in the entire concentration range of the calibration curve.

The SPE procedure described earlier [9] used 0.5 mL serum, 0.5 mL saturated sodium chloride solution, 1 mL acetonitrile and 8 mL chloroform with total final volume of 10 mL. This large volume practically caused some difficulties during mixing of samples. In addition, in some cases creation of emulsion prevented the separation of aqueous and organic phase. Furthermore, Conte *et al.* [5] method resulted in peak broadening. A 40 % acetonitrile in water was employed to achieve the resolution. It reduced the retention time of internal standard (IS) and ETA peaks resulting due to the interference of the latter and endogenous serum peaks. The same behavior was observed when the flow rate was adjusted below 0.9 mL/min. So the modifications in the previous methods resulted in good separation and isolation peaks resulted in simple and sensitive method for determination of ETA in serum.

Experimental

Chemicals and Reagents

Ethionamide hydrochloride was imported from Organisynth Laboratories India. Disodium hydrogen phosphate and HPLC grade acetonitrile were purchased form Merck (Germany). All other chemicals and solvents were of HPLC/ analytical grade.

Solutions and Buffers

Stock solution (100 μg/ mL) of ETA was prepared by dissolving 10 mg drug in 100 mL of acetonitrile. Standard solutions were obtained by diluting this solution with acetonitrile to give concentration over the range of 0.1 to 4.0 μg/ mL.

Mobile Phase

Mobile phase consisted of disodium hydrogen phosphate 0.02 M and acetonitrile (75: 25, v/ v). Disodium hydrogen phosphate was prepared by dissolving 3.588 g in 200 mL of distilled water and mixing thoroughly until complete solubility and making the volume 1000 mL. It was filtered under vacuum using 0.45 μm membrane filter (Sartorius) and degassed on sonicator for 3 min.

Chromatographic Condition

Analysis was performed on Shimadzu liquid chromatography, with a pump LC 10 A.S, SPD 10A Shimadzu, U.V. detector at 291 nm. A reverse phase system was used consisting of a 5 μm hypersil ODS C_{18} column (250 mm x 4.6 mm) preceded by 5-μm hypersil ODS C_{18} cartridge guard (10 mm x 4.6 mm) column.

Extraction and Quantification

The standard dilutions were made from the stock solution in the range of 0.1-4 μg/ mL. One mL serum and 1 mL of each of standard dilution was mixed and centrifuged at 4000 rpm for 10 min. The supernatant layer was removed and filtered through Millipore filtration assembly. Filtrate was transferred to the polypropylene tube and evaporated under constant stream of nitrogen at 50 °C using sample concentrator. The residue was reconstituted with acetonitrile 100 μl and 60 μL was injected into HPLC system.

Calibration curves were constructed by plotting the peak area of ETA vs concentration. The intra day (within-run) and inter day (between run) accuracy and precision were determined by measuring ETA standard solution on three separate days.

Conclusions

A single step extraction procedure for ETA from serum and an improved method for the determination of ETA is reported. Compared to previously published methods, the suggested extraction procedure is considerably simple, rapid, reliable, reproducible and sensitive. The HPLC technique based on isocratic system and U.V. detection makes this method suitable to determine small amounts of ETA with good accuracy and reproducibility comparable to the methods performed by gradient reserved phase liquid chromatography with U.V. detection and fluo-

rescence detection. Simple sample preparation procedure and a short chromatographic time make this method suitable for processing of multiple samples in a limited time for bioequivalence and pharmacokinetic studies.

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